Attorney's Docket No 01-057001 / C2-906DP1PCT-US

APPLICATION

FOR

UNITED STATES LETTERS PATENT

TITLE: TRANSPORTER GENES

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TRANSPORTER GENES

This application is a continuation-in-part of PCT/JP98/04009 filed September 7, 1998, and claims priority from Japanese Application No. 9/260972, filed September 8, 1997 and Japanese Application No. 10/156660, filed May 20, 1998.

Field of the Invention

The present invention relates to transporters, proteins involved in transport of substances from the outside to the inside of cells or vice versa.

Background of the Invention

Recently, the involvement of various transporters localized on the plasma membrane in the uptake system for nutrients and endogenous substances into cells and their transport mechanisms have been clarified (Tsuji, A. and Tamai, I., Pharm. Res., 13, 963-977, 1996). These transporters recognize the structures of substances to be transported to selectively transport specific substances. Transporters that recognize the relatively wide range of structures may possibly recognize foreign substances such as drugs by mistake, and actively take in them into cells. It is believed that drugs permeate through the plasma membrane fundamentally by simple diffusion depending on their physicochemical properties such as molecular size, hydrophobicity, and hydrogen-binding capacity. Particularly, in the case of ionic drugs, only molecules in the non-dissociated form can permeate through the plasma membrane according to the pH partition hypothesis.

However, it has become evident that a number of drugs penetrate through the cell membrane by a specific mechanism other than simple diffusion, that is, an active transport mediated by transporters, in organs that require efficient exchange of intracellular and extracellular substances, including small intestine, uriniferous tubule, placenta, epithelial cells of choroid plexus, hepatocytes, and blood-brain barrier (Tamai, I. and Tsuji, A., Pharmacia, 31, 493-497, 1995; Saito, H. and Inui, K., Igaku no Ayumi, 179, 393-397, 1996; Tamai, I., Yakubutsu Dotai (Pharmacokinetics), 11, 642-650, 1996). For example, it is known that although oral β-lactam antibiotics of the non-esterified type are amphoteric or negatively charged in physiological pHs and sparingly soluble in fat, they are readily absorbed through the intestine. The transport study using the isolated membrane-vesicles system demonstrated that an H^+-driven peptide transporter localized on the brush-border is involved in the absorption process of these drugs (Tsuji, A. et al., J. Pharmacol. Exp. Ther., 241, 594-601, 1987). Although the specificity of a peptide transport system in terms of the peptide size is so strict as to recognize di- or tri-peptides but not tetrapeptides or larger peptides, it has a rather broad substrate specificity to recognize peptides comprising non-natural amino acids. The peptide transporter mediates transport of β-lactam antibiotics mistakenly due to its broad substrate specificity. This property has been unexpectedly utilized in the clinical field (Tsuji, A., American Chemical Society (eds. Taylor, M. D., Amidon, G. L.), Washington, D. C., 101-134, 1995). Furthermore, a possibility that a transporter is also involved in permeation of substances

with a high hydrophobicity such as fatty acids through the plasma membrane has been reported (Schaffer, J. and Lodish, H., Cell, 79, 427-436, 1994).

Since various transporters are supposed to be distributed in organs and cells based on the physiological roles of the organs and cells, their distribution and functions may be specific to organs. Therefore, transporters are expected to be used to impart an organ specificity to pharmacokinetics. In other words, an organ-specific drug delivery system (DDS) can be constructed utilizing transporters. If drug absorption solely relied on simple diffusion is improved by elevating its hydrophobicity, an effect of the drug obtained in the initial transport in the liver can be enhanced and the drug can non-specifically translocates into any organ. In addition, it would also be possible to increase the drug absorption independently of its fat-solubility by designing the drug utilizing the substrate specificity of transporters (Hayashi, K. et al., Drug Delivery System, 11, 205-213, 1996). For this purpose, it is necessary to identify various transporters at the molecular level and analyze their properties in detail. However, their molecular level identification are greatly behind studies on their membrane physiology because they are difficult to handle biochemically and require complicated processes in their functional assays.

Recently, cDNAs of several transporters have been cloned by the expression cloning method using *Xenopus* oocytes, a foreign gene expression system, and structural homology among them has been revealed (Fei, Y.-J. et al., Nature, 368, 563-566, 1994). For example, Koepsell et al. cloned

an organic cation transporter, OCT1, which is assumed to be localized on a basement membrane, using the expression cloning method in 1994 (Grundemann, D. et al., Nature, 372, 549-552, 1994). Subsequently, OCT2 was identified by homology cloning based on the sequence of OCT1 (Okuda, M. et al., Biochem. Biophys. Res. Commun., 224, 500-507, 1996). OCT1 and OCT2 show homology as high as 67% to each other (Grundemann, D. et al., J. Biol. Chem., 272, 10408-10413, 1997). Both of them are intensely expressed in the kidney, but differ in the organ distribution; OCT1 is also expressed in the liver, colon, and small intestine, while OCT2 expression is specific to the kidney.

Only a few reports on identification of transporters at the molecular level, including the reports, are available, and there would be many unknown transporters that may be clinically useful.

Summary of the Invention

An object of this invention is to provide a family of novel transporter genes, proteins encoded by these genes, and their use.

The present inventors have screened a fetal gene library constructed using the subtractive method by random sequencing based on a working hypothesis that fetal genes include those which are involved in various disorders including cancer and are specifically or intensely expressed in fetal tissues. The inventors discovered an unknown gene showing a significant homology with those for organic cation transporters, OCT1 and OCT2, and attempted to isolate this gene, which was assumed to encode a novel

transporter. Thus, the inventors succeeded in isolating the desired gene by screening a cDNA library derived from human fetus. Furthermore, the inventors studied the transporter activity of a protein encoded by the isolated human gene and found that the protein, in fact, functioned as a transporter for various organic cations. The inventors also succeeded in isolating a mouse gene corresponding to the isolated human gene.

This invention relates to a family of novel transporter genes, proteins encoded by these genes, and their use, and more specifically to:

(1) a protein comprising an amino acid sequence set forth in SEQ ID NOs: 1, 3, 22, or 27, or a protein comprising said amino acid sequence in which one or more amino acid residues are substituted, deleted, or added, and having an activity to transport an organic cation;

(2) a protein encoded by a DNA hybridizing to a DNA comprising nucleotide sequence according to SEQ ID NOs: 2, 4, 23, or 28, and having an activity to transport an organic cation;

(3) a DNA encoding the protein according to (1) or (2);

(4) a vector comprising the DNA according to (3);

(5) a transformant expressibly carrying the DNA according to (3);

(6) a method for producing the protein according to (1) or (2), the method comprising culturing the transformant according to (5);

(7) an antibody that binds to the protein according to (1) or (2); and

(8) a DNA specifically hybridizing to a DNA comprising a nucleotide sequence set forth in SEQ ID NOs: 2, 4, 23, or 28, and consisting of at least 15 nucleotides.

Nucleotide sequences of cDNAs of novel human transporters isolated by the present inventors are shown in SEQ ID NO: 2 (designated as "human OCTN1") and SEQ ID NO: 4 (designated as "human OCTN2"), respectively. Amino acid sequences of proteins encoded by these cDNAs are shown in SEQ ID NO: 1 and SEQ ID NO: 3, respectively. Amino acid sequences of these two proteins included in the transporter proteins of this invention showed such a high overall homology as about 76%, and both of them retained the following consensus sequence which is conserved in various types of transporters including the glucose transporter: [Leu, Ile, Val, Met, Ser, Thr, Ala, Gly]-[Leu, Ile, Val, Met, Phe, Ser, Ala, Gly]-Xaa<2>-[Leu, Ile, Val, Met, Ser, Ala]-[Asp, Glu]-Xaa-[Leu, Ile, Val, Met, Phe, Tyr, Trp, Ala]-Gly-Arg-[Arg, Lys]-Xaa<4-6>-[Gly, Ser, Thr, Ala] (Maiden, M. C. et al., Nature, 325, 641-643, 1987). In fact, these proteins have an activity to transport various organic cations (see Examples 6 to 8).

The present inventors also isolated mouse genes corresponding to the above-described human OCTN1 and human OCTN2. Nucleotide sequences of the isolated cDNAs are shown in SEQ ID NO: 23 (designated as "mouse OCTN1") and SEQ ID NO: 28 (designated as "mouse OCTN2"), respectively. Amino acid sequences of proteins encoded by these cDNAs are shown in SEQ ID NOs: 22 and 27, respectively.

Transporter proteins of this invention also include those having the additional activity to transport substances other than organic cations as far as they retain the organic cation transport activity. Organic cations include, for example, TEA, carnitine, quinidine, and pyrilamine, but are not limited to them. They also include carcinostatic agents such as actinomycin D, etoposide, vinblastine, daunomycin, etc. Transporter proteins of this invention include those having the activity to transport organic cations not only from the outside to the inside of cells but also from the inside to the outside of cells.

Transporter proteins of this invention can be prepared as recombinant proteins using recombination techniques or natural proteins. Recombinant proteins can be prepared, for example, as described below, by culturing cells transformed with DNA encoding proteins of this invention. Natural proteins can be isolated from the kidney and cancer cell strains such as Hela S3, which highly express the proteins of this invention, by the method well known to those skilled in the art, for example, affinity chromatography using an antibody of this invention described below. The antibody may be either polyclonal or monoclonal. A polyclonal antibody can be prepared by purifying serum obtained from, for example, a small animal such as a rabbit immunized with proteins of this invention by known methods, for example, ammonium sulfate precipitation, protein A or protein G column, DEAE-ion exchange column chromatography, affinity column chromatography coupled with the protein of this invention, etc. A monoclonal antibody can be prepared by immunizing a small animal such as a mouse with the protein of this

invention, excising the spleen from the mouse, grinding the tissue into cells, fusing them with mouse myeloma cells using a fusing agent such as polyethylene glycol, and selecting a clone that produces an antibody to proteins of this invention out of fused cells (hybridomas) thus produced. Then, hybridomas thus selected are transplanted into the abdominal cavity of a mouse, and the ascites is collected from the mouse. A monoclonal antibody thus obtained can be purified by, for example, ammonium sulfate precipitation, protein A or protein G column, DEAE-ion exchange column chromatography, affinity column chromatography coupled with the protein of this invention, etc. When the antibody thus obtained is administered to human subjects, a humanized antibody or a human antibody is advantageously used to reduce the immunogenicity. An antibody can be humanized by, for example, the CDR grafting method comprising cloning an antibody gene from monoclonal antibody-producing cells and grafting the epitope portion thereof into an existing human antibody. A human antibody can be prepared by the usual method for preparing a monoclonal antibody except for immunizing a mouse whose immune system is replaced with the human's.

The term "substantially pure" as used herein in reference to a given polypeptide means that the polypeptide is substantially free from other biological macromolecules. The substantially pure polypeptide is at least 75% (e.g., at least 80, 85, 95, or 99%) pure by dry weight. Purity can be measured by any appropriate standard method, for example, by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A "conservative amino acid substitution" is one in which an amino acid residue is replaced with another residue having a chemically similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

It is also possible for those skilled in the art to prepare proteins having functions equivalent to the transporter proteins of this invention (human OCTN1, human OCTN2, mouse OCTN1, and mouse OCTN2) by appropriately modifying amino acid residues of the proteins by, for example, substitution, using well known methods. Mutation of amino acids of the proteins can occur also spontaneously. Such mutant proteins which are obtained by altering the amino acid sequence of the transporter proteins of this invention by substitution, deletion, or addition of amino acid residues, and are functionally equivalent to those of the transporter proteins are also included in the proteins of this invention. Herein, "functionally equivalent" means that proteins have an activity to transport organic cations. Methods well known to those skilled in the art for altering amino acids include, for example, the site-specific mutagenesis system

by PCR (GIBCO-BRL, Gaithersburg, Maryland), site-specific mutagenesis by oligonucleotide (Kramer, W. and Fritz, H. J. (1987) Methods in Enzymol., 154: 350-367), Kunkel's method (Methods Enzymol., 85, 2763-2766 (1988)), etc. The number of amino acids that can be substituted is usually 10 amino acid residues or less, preferably 6 or less, and more preferably 3 or less. The site of substitution, deletion, or addition of amino acid residues is not particularly limited as far as the activity of proteins of this invention is retained. It is possible to detect the transporter activity of proteins, for example, by the method described below in Example 6.

It is routine for those skilled in the art to obtain proteins functionally equivalent to the transporter proteins of this invention by isolating and using DNAs highly homologous to the DNA sequences encoding the transporter proteins of this invention (human OCTN1, human OCTN2, mouse OCTN1, and mouse OCTN2) or portions thereof using hybridization techniques (Sambrook, J. et al., Molecular Cloning 2nd ed., 9.47-9.58, Cold Spring Harbor Lab. press, 1989), etc. These proteins functionally equivalent to those of transporter proteins of this invention are also included in proteins of this invention. Here, "functionally equivalent" means that proteins have an activity to transport organic cations. DNAs that hybridize to the DNAs encoding the proteins of this invention can be isolated from other organisms, for example, rats, rabbits, cattle, etc. as well as humans and mice. Especially, tissues such as the kidney are suitable as sources of such DNAs. These DNAs isolated using hybridization techniques usually have a high homology with the above-described DNAs

encoding the transporter proteins of this invention. "High homology" means at least 70% or more, preferably at least 80% or more, and more preferably at least 90% or more of amino acid sequence identity. The "percent identity" of two amino acid sequences or of two nucleic acids is determined using the algorithm of Karlin and Altschul (Proc. Natl. Acad. Sci. USA 87:2264-2268, 1990), modified as in Karlin and Altschul (Proc. Natl. Acad. Sci. USA 90:5873-5877, 1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul et al. (J. Mol. Biol. 215:403-410, 1990). BLAST nucleotide searches are performed with the NBLAST program, score = 100, wordlength = 12. BLAST protein searches are performed with the XBLAST program, score = 50, wordlength = 3. Where gaps exist between two sequences, Gapped BLAST is utilized as described in Altschul et al. (Nucleic Acids Res. 25:3389-3402, 1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used. See http://www.ncbi.nlm.nih.gov.

One example of hybridization conditions for isolating such DNAs is as follows. That is, after the pre-hybridization at 55°C for 30 min or more in the "ExpressHyb Hybridization Solution" (CLONTECH), a labeled probe is added, and hybridization is performed by heating the reaction mixture at 3°C to 55°C for 1 h or more. Then, the reaction product is successively washed in 2 x SSC and 0.1% SDS three times at room temperature for 20 min, and then in 1 x SSC and 0.1% SDS once at 37°C for 20 min. More preferable conditions are as follows. After the pre-hybridization at 60°C for 30 min or more in the "ExpressHyb

Hybridization Solution" (CLONTECH), a labeled probe is added, and hybridization is performed by heating the reaction mixture at 60°C for 1 h or more. Then, the reaction product is successively washed in 2 x SSC and 0.1% SDS three times at room temperature for 20 min, and then in 1 x SSC and 0.1% SDS twice at 50°C for 20 min. Still more preferable conditions are as follows. After pre-hybridization at 68°C for 30 min or more in the "ExpressHyb Hybridization Solution" (CLONTECH), a labeled probe is added, and hybridization is performed by heating the reaction mixture at 68°C for 1 h or more. Then, the reaction product is successively washed in 2 x SSC and 0.1% SDS three times at room temperature for 20 min, and then in 0.1 x SSC and 0.1% SDS twice at 50°C for 20 min.

The present invention also relates to DNAs encoding the above-described transporter proteins of this invention. DNAs of this invention may be cDNA, genomic DNAs, and synthetic DNAs. The DNAs of the present invention can be used for producing proteins of this invention as recombinant proteins. That is, it is possible to prepare proteins of this invention as recombinant proteins by inserting DNAs encoding proteins of this invention (e.g. DNAs comprising the nucleotide sequences set forth in SEQ ID NOs: 2, 4, 23, and 28) into an appropriate expression vector, culturing transformants obtained by transfecting suitable cells with the vector, and purifying the proteins thus expressed. Cells to be used for producing recombinant proteins include, for example, mammalian cells such as COS cells, CHO cells, NIH3T3 cells, etc., insect cells such as Sf9 cells, yeast cells, *E. coli*, and so on. Vectors used for the intracellular expression of recombinant proteins

vary depending on host cells, including, for example, pcDNA3 (Invitrogen), pEF-BOS (Nucleic Acids Res., 1990, 18(7), p5322), etc. for mammalian cells, "BAC-to-BAC baculovirus expression system" (GIBCO BRL), etc. for insect cells, "Pichia Expression Kit" (Invitrogen), etc. for yeast cells, pGEX-5X-1 (Pharmacia), "QIAexpress system" (Qiagen), etc. for *E. coli*. Host cells can be transformed with vectors, for example, by the calcium phosphate method, the DEAE-dextran method, the method using cationic liposome DOTAP (Boehringer Mannheim), the electroporation method, the calcium chloride method, etc. Recombinant proteins can be purified from recombinants thus obtained using standard methods, for example, as described in "The Qiaexpressionist Handbook, Qiagen, Hilden, Germany."

The present invention also relates to DNAs consisting of at least 15 nucleotides that specifically hybridize to the DNAs encoding proteins of this invention. Herein, "specifically hybridize" means that a DNA does not cross-hybridize to other DNAs encoding other proteins under usual hybridization conditions, preferably under the stringent hybridization conditions. Such a DNA can be utilized as a probe for detecting and isolating DNA encoding the protein of this invention, and as a primer for amplifying the DNA.

By hybridization under "stringent conditions" is meant hybridization at 37°C, 1 X SSC, followed by washing at 42°C, 0.5 X SSC.

An "isolated nucleic acid" is a nucleic acid the structure of which is not identical to that of any naturally occurring nucleic acid or to that of any fragment of a naturally occurring genomic nucleic acid spanning more than three separate genes. The term therefore covers, for

example, (a) a DNA which has the sequence of part of a naturally occurring genomic DNA molecule but is not flanked by both of the coding sequences that flank that part of the molecule in the genome of the organism in which it naturally occurs; (b) a nucleic acid incorporated into a vector or into the genomic DNA of a prokaryote or eukaryote in a manner such that the resulting molecule is not identical to any naturally occurring vector or genomic DNA; (c) a separate molecule such as a cDNA, a genomic fragment, a fragment produced by polymerase chain reaction (PCR), or a restriction fragment; and (d) a recombinant nucleotide sequence that is part of a hybrid gene, i.e., a gene encoding a fusion protein. Specifically excluded from this definition are nucleic acids present in mixtures of different (i) DNA molecules, (ii) transfected cells, or (iii) cell clones: e.g., as these occur in a DNA library such as a cDNA or genomic DNA library.

The transporter proteins of this invention can be used to control internal absorption and dynamics of drugs. Based on the results of detailed analysis of the substrate specificity of transporter proteins of this invention, drugs can be designed so as to be transported by these transporters and absorbability of the drugs mediated by these transporter proteins can be improved. Conventional modifications to enhance hydrophobicity are no longer necessary for drugs so designed, which enables speedily and efficiently developing water-soluble drugs that are easy to handle. The drugs thus developed is thought to be absorbed principally depending on the internal distribution pattern of transporter proteins of this invention, and an organ-specific delivery of the drugs thus becomes possible.

Especially, if the transporter proteins of this invention are distributed in the target organ of a drug, an ideal drug delivery system (DDS) can be developed. If a drug is to be absorbed mediated by not the transporter proteins of this invention but other transporters, the drug can be designed so as to be specific to other transporter proteins by designing it considering the substrate specificity of the transporter proteins of this invention. Since the transporter proteins of this invention are present in the kidney, it is possible to reduce the nephrotoxicity produced by a drug by designing the drug so that it can be readily excreted by the transporter proteins of this invention.

Another possible application of this invention is to develop a drug targeting the transporter proteins of this invention. The transporters play important roles in the absorption mechanism of nutrients and drugs, or the excretion mechanism of drugs and internal metabolites. Thus, damage or abnormal elevation of the transporter's functions may cause some disorders. It is considered to be efficacious against such disorders to administer a drug containing a compound that inhibits or enhances functions of the transporter proteins of this invention, or regulates the expression level of the transporter gene of this invention and the amount of the transporter proteins. The DNAs of this invention can be used in gene therapy for disorders caused by abnormalities in the activity and expression of the proteins of this invention. In this case, the DNA of this invention are inserted to an adenovirus vector (e.g. pAdexLcw), a retrovirus vector (e.g. pZIPneo), etc., and administered into the body by

either *ex vivo* method or *in vivo* method. Gene therapy can also be performed by administering a synthetic antisense DNA to the body either directly or after inserted into the above-described vector.

Especially, since "OCTN2" included in the transporter proteins of this invention efficiently transports carnitine, chemotherapy with compounds to control the activity of "OCTN2" or gene therapy using the "OCTN2" gene is considered to be efficacious against various pathological conditions such as fatty liver, myocardiopathy, myopathy, etc. caused by hypocarnitinemia.

The transporter proteins of this invention are expressed in a variety of cancer cell strains, which suggests that the proteins may transport drugs into tumor cells. If this is the case, it is possible to develop carcinostatics that will be readily absorbed mediated by the transporter proteins of this invention. On the contrary, mechanisms to transport and excrete substances by the transporter proteins of this invention may function to excrete carcinostatics in tumor cells so that the cells acquire resistance to drugs. If the transporter proteins of this invention are involved in a mechanism of tumor cells to acquire drug resistance, a carcinostatic effect can be enhanced by a combined use of inhibitors of the transporter proteins of this invention with carcinostatics.

Brief Description of the Drawings

Fig. 1 represents hydrophobicity plots of human OCTN1 and human OCTN2 according to Kyte & Doolittle's calculating formula with a window of nine amino acid residues.

Numerals on the plots indicate putative transmembrane regions.

Fig. 2 represents electrophoretic patterns showing the results of Northern blot analysis of human OCTN1.

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~~Fig. 3 compares the amino acid sequence of human OCTN1 with that of human OCTN2. Amino acid residues conserved in both transporters are shaded. Sequences coinciding with the consensus sequences of sugar transporter and the ATP/GTP binding site are indicated by "+" and "*," respectively.~~

Fig. 4 represents electrophoretic patterns showing the results of Northern blot analysis of human OCTN2.

Fig. 5 is a graph showing the TEA-absorbing activity of human OCTN1. Clear circles represent untreated cells, and solid circles represent human OCTN1-transfected cells.

Fig. 6 is a graph showing effects of the cold TEA added in the experimental system in Fig. 5. In this graph, solid circles represent human OCTN1-transfected cells, and clear circles represent cells containing the vector with no insert. Clear triangles indicate the net uptake induced by human OCTN1 obtained by subtracting the clear circle values from the corresponding solid circle values.

Fig. 7 is a graph showing TEA concentration-dependency of the TEA-absorbing activity of human OCTN1.

Fig. 8 is a bar graph showing the activity of the human OCTN1-transfected cells to absorb substances other than TEA.

Fig. 9 is a bar graph showing the results of transport experiments using *Xenopus* oocytes. Bars indicated with

"OCTN1" and "Water" represent the uptake activity of the human OCTN1-injected cRNA oocytes and that of the water-injected oocytes (containing no cRNA), respectively. Uptakes of TEA, carnitine, mepyramine, quinidine, and actinomycin D were observed in human OCTN1 cRNA-injected oocytes, whereas water-injected oocytes (containing no cRNA) exhibited almost no uptake activity.

Fig. 10 is a bar graph showing the results of transport experiments for carcinostatics in *Xenopus* oocytes. Bars indicated with "OCTN1" and "Water" represent the uptake activity of the human OCTN1 cRNA-injected oocytes and that of the water-injected oocytes (containing no cRNA), respectively. Uptakes of actinomycin D, etoposide, vinblastine, and daunomycin were observed in the human OCTN1 cRNA-injected oocytes.

Fig. 11 is a bar graph showing the results of transport experiments with human OCTN1 and human OCTN2 in HEK293 cells. Human OCTN1 has the efficient transport activity for TEA and human OCTN2 for carnitine.

Fig. 12 is a graph showing the results of Na^+-dependency of the carnitine transport activity of human OCTN2. Human OCTN2 exhibits a time-dependent carnitine transport activity (clear circle) in the presence of Na^+, while no such activity in the absence of Na^+ (solid circle), indicating that the carnitine transport activity of human OCTN2 depends on the presence of Na^+.

Fig. 13 shows the expression of mouse OCTN1 and mouse OCTN2 genes detected by RT-PCR amplification in each tissue. G3PDH serves as a control, indicating that the amount of cDNA in each tissue is uniform.

Detailed Description

The present invention is described below in more detail with reference to examples, but is not construed being limited thereto.

Example 1 Construction of a subtraction library

A subtraction library was constructed using the PCR-Select™ cDNA Subtraction Kit (CLONTECH) principally according to the method of Luda Diatchenko (Diatchenko, L. et al., Proc. Natl. Acad. Sci. USA, 93, 6025-6030, 1996).

First, double-stranded cDNAs were synthesized from poly(A)$^+$ RNAs derived from human fetal liver and adult liver by the standard method using MMLV reverse transcriptase. These cDNAs were blunt-ended with T4 DNA polymerase and cleaved with RsaI. A part of the cDNAs derived from fetal liver (tester) was divided in two portions, and they were separately ligated to two different adapters, adapter 1 and adapter 2, respectively (Table 1). A 120-fold excess of cDNA derived from adult liver (driver) was added to each of the above-described tester samples. The mixture was heat-denatured and subjected to the primary hybridization at 68°C for 8 h. After these two reaction mixtures from the primary hybridization were mixed together without heat-denaturation, an excessive amount of the heat-denatured driver was further added thereto, and the mixture was subjected to the secondary hybridization at 68°C for about 16 h. The resulting reaction solution was diluted with a dilution buffer and incubated at 75°C for 7 min. After the shorter strands of adapters were removed, the reaction

solution was used as a template for PCR. PCR using primers 1 (5'-CTAATACGACTCACTATAGGGC-3', SEQ ID NO: 5) and 2 (5'-TGTAGCGTGAAGACGACAGAA-3', SEQ ID NO: 6) corresponding to the adapters selectively amplified only cDNAs having different adapters at their both ends (subtracted cDNAs)(suppression PCR). PCR was carried out using a portion of the resulting cDNA as a template, and nested PCR primers 1 (5'-TCGAGCGGCCGCCCGGGCAGGT-3', SEQ ID NO: 7) and 2 (5'-AGGGCGTGGTGCGGAGGGCGGT-3', SEQ ID NO: 8), which are further inwardly located from the PCR primers 1 and 2, to obtain products with further elevated selectivity. PCR products thus obtained were purified using the QIAquick PCR Purification kit (QIAGEN), and cloned into the pT7Blue-T vector (Novagen) by the TA cloning method to construct a subtraction library.

Table 1

Adapter 1	5'-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3' 3'-GGCCCGTCCA-5'
Adapter 2	5'-TGTAGCGTGAAGACGACAGAAAGGGCGTGGTGCGGAGGGCGGT-3' 3'-GCCTCCCGCCA-5'

The longer strand of the partially single stranded DNA of Adapters 1 and 2 are designated SEQ ID NOs:29 and 30, respectively, and the shorter strand of Adapters 1 and 2 are designated SEQ ID NOs:31 and 32, respectively.

Example 2 cDNA cloning

To analyze fetal genes, the subtraction library derived from the fetal liver was screened by random sequencing. Homology search (Blastx) of Expressed Sequence Tags (ESTs) thus obtained found a clone, OCTN1 (fls 631) (292 bp) encoding amino acid sequence having significant homology with the known organic cation transporters, OCT1 (Grundemann, D. et al., Nature, 372, 549-552, 1994) and OCT2 (Okuda, M. et al., Biochem. Biophys. Res. Commun., 224, 500-507, 1996). Since the sequence of this clone was novel and assumed to be a fragment derived from a new transporter gene, cDNA comprising the whole open reading frame (ORF) of this gene was cloned.

The human fetal liver 5'-stretch cDNA library (CLONTECH) was screened using the original OCTN1 clone obtained from the subtraction library derived from fetal liver as a probe. An insert of the original OCTN1 clone was amplified by PCR using M13 P4-22 and M13 P5-22, and labeled with [α-^{32}P]dCTP by the random primer method using the Ready-to Go DNA labeling beads (Pharmacia) to serve as a probe. Hybridization was carried out at 68°C in the ExpressHyb Hybridization Solution (CLONTECH) according to the method recommended by the manufacturer. Final washing was performed at 50°C in 0.1 x SSC and 0.1% SDS. Screening about 5 x 10^5 phage clones finally isolated seven positive clones. cDNA inserts of these clones were amplified by PCR using vector primers designed based on a sequence of the λgt10 vector (GT10 S1 5'-CTTTTGAGCAAGTTCAGCCT-3', SEQ ID NO: 9, and GT10 A1 5'-AGAGGTGGCTTATGAGTATTTCTT-3', SEQ ID NO: 10), or primers designed based on the decoded cDNA sequences. The PCR products thus obtained were directly

sequenced to determine the nucleotide sequences. Some regions that were difficult to be amplified were subjected to PCR using 7-deaza dGTP as a substrate base (McConlogue, L. et al., Nucleic Acids Res., 16, 9869, 1988).

Sequencing of cDNA inserts of these clones revealed that the human OCTN1 gene contains an ORF encoding a protein consisting of 551 amino acid residues (putative molecular weight of about 62,000). Data base search using this whole amino acid sequence confirmed that it has a significant overall homology (about 34%) with OCT1 and OCT2. Hydrophobicity profile of this sequence obtained by Kyte & Doolittle's calculating formula (Kyte, J. and Doolittle, R. F., J. Mol. Biol., 157, 105-132, 1982) very closely resembled those of OCT1 and OCT2, indicating that the sequence has eleven to twelve putative transmembrane hydrophobic regions (Fig. 1). This sequence contained one consensus sequence of sugar transporter, ([Leu, Ile, Val, Met, Ser, Thr, Ala, Gly]-[Leu, Ile, Val, Met, Phe, Ser, Ala, Gly]-Xaa<2>-[Leu, Ile, Val, Met, Ser, Ala]-[Asp, Glu]-Xaa-[Leu, Ile, Val, Met, Phe, Tyr, Trp, Ala]-Gly-Arg-[Arg, Lys]-Xaa<4-6>-[Gly, Ser, Thr, Ala]), (160 to 175). This consensus sequence is present in the glucose transporters GLUT1 to GLUT7 in mammalian cells, and also present in various types of transporters other than glucose transporters (Maiden, M. C. et al., Nature, 325, 641-643, 1987). Furthermore, putative N-linked glycosylation sequences (N-X-[ST]) were found in the amino acid sequence of human OCTN1 at four sites (57 to 59, 64 to 66, 91 to 93, and 304 to 306), and also five putative protein kinase C phosphorylation sites ([ST]-X-[RK]) (164 to 166, 225 to 227, 280 to 282, 286 to 288, and 530 to 532). In addition,

the consensus sequence ([Ala, Gly]-Xaa(4)-Gly-Lys-[Ser, Thr]) of the ATP/GTP binding site is also found. This consensus sequence of the ATP/GTP binding site is also present in the ATP binding protein or GTP binding protein, such as kinases and ras family proteins, and that ATP or GTP binds to this site (Walker, J. E. et al., EMBO J., 1, 945-951, 1982). This sequence is present in the so-called ATP Binding Cassette (ABC) type transporter, and involved in the substance transport using the energy generated by hydrolysis of ATP (Higgins, C. F. et al., J. Bioenerg. Biomembr., 22, 571-592, 1990; Urbatsch, I. L. et al., J. Biol. Chem., 270, 26956-26961, 1995). Presence of this consensus sequence indicates that OCTN1 protein may be an ATP or GTP-dependent transporter.

Nucleotide sequencing was performed by the cycle-sequencing method with a plasmid DNA prepared by the alkaline-SDS method or a PCR product obtained by colony PCR, etc. as a template using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit With AmplyTaq DNA Polymerase, FS, followed by decoding with the ABI 377 DNA Sequencer (Perkin Elmer). Colony PCR was carried out by directly suspending a colony of a recombinant in a PCR reaction solution containing vector primers M13 P4-22 (5'-CCAGGGTTTTCCCAGTCACGAC-3', SEQ ID NO: 11) and M13 P5-22 (5'-TCACACAGGAAACAGCTATGAC-3', SEQ ID NO: 12). After the completion of PCR, a DNA insert thus amplified was separated from unreacted primers and nucleotides by gel filtration, etc. to serve as a template for sequencing.

Example 3 Northern analysis

Distribution of human OCTN1 in tissues was investigated by Northern analysis (Fig. 2). A 3'-end fragment of human OCTN1 (the latter half from around the base 1,100) was labelled with [α-^{32}P]dCTP by the random primer method using the Ready-to Go DNA labeling beads (Pharmacia) to serve as a probe. Hybridization was performed using the Multiple Tissue Northern (MTN) Blot - Human, Human III, Human IV, Human Fetal II, and Human Cell lines (CLONTECH) at 68°C in the ExpressHyb Hybridization Solution (CLONTECH) according to the method recommended by the manufacturer. Final washing was performed at 50°C in 0.1 x SSC and 0.1% SDS. As a result, RNA of about 2.5 kb was strongly expressed in the fetal liver and adult-derived tissues such as the kidney, bone marrow, and trachea. Besides those tissues, the RNA band was also weakly detected in the fetal kidney and lung, and adult tissues including skeletal muscle, lung, placenta, prostate, spleen, and spinal cord. The RNA expression was also detected in tumor cell lines such as HeLa S3, K562, SW480, and A549, and especially, its very intense expression was observed in HeLa S3.

Example 4 Cloning of human OCTN2 cDNA

Data base search using the entire nucleotide sequence of "human OCTN1" can detect very similar sequences thereto in several parts of the nucleotide sequence of P1 phage clones (P1 H24 clones, GenBank accession No. L43407, L43408, L46907, L81773, and L43409) derived from q regions of human chromosome 5. The parts having similarity with the nucleotide sequence of human OCTN1 are separated by the

sequences having no similarity to the human OCTN1 sequence. The sequence obtained by connecting these similar parts with each other with reference to the sequence of human OCTN1 has a high homology over a wide range with human OCTN1, indicating the presence of OCTN1 homologues. The genomic sequence registered in data base was an incomplete one without covering the entire coding region, and, from only this sequence, it was impossible to know the complete structure of a protein partially encoded by the sequence. Therefore, cDNA cloning of this OCTN1 homologous gene (OCTN2) was performed to determine the coded protein structure. First, 631R S4 primer (5'-GTGCTGTTGGGCTCCTTCATTTCA-3', SEQ ID NO: 13) and 631R A1 primer (5'-AGCTGCATGAAGAGAAGGACACTG-3', SEQ ID NO: 14) were prepared based on sequences of these P1 phage clones. PCR was performed using a set of these primers and cDNA synthesized from poly(A)$^+$ RNA derived from the human adult kidney (CLONTECH) as a template, under the following conditions: 1 cycle of 94°C for 3 min; 35 cycles of 94°C for 30 s, 58°C for 1 min, and 72°C for 2 min,; and 1 cycle of 72°C for 10 min, resulting in amplification of about 900 bp fragment. This fragment was subcloned into the pT7Blue-T vector (Novagen) by the TA cloning method to determine its nucleotide sequence, which clearly showed a very high overall homology with human OCTN1. Therefore, this gene was designated as human OCTN2, and longer cDNAs were cloned.

The cDNA library derived from the human kidney was screened using the cDNA insert of this clone as a probe in the same manner as for human OCTN1 cDNA cloning, and cDNA containing the entire coding region of human OCTN2 was

cloned by a procedure for isolating longer clone and the Rapid Amplification of cDNA Ends (RACE) method (Chenchik, A., Moqadam, F., and Siebert, P. (1995), CLONTECHniques X, 5-8), etc. to determine its structure (SEQ ID NO: 4). Specifically, the RACE method was carried out as follows. The 631R S6 primer (5'-AGCATCCTGTCTCCCTACTTCGTT-3', SEQ ID NO: 15) was prepared. PCR was performed using this primer and the Marathon-Ready™ cDNA derived from the human adult kidney (CLONTECH) as a template under the following conditions: 94°C for 2 min; 35 cycles of 94°C for 30 s, 60°C for 1 min, and 72°C for 3 min,; and 72°C for 10 min, resulting in amplification of about 1.7 kbp cDNA fragment of the 3'-end. This fragment was subcloned into the pT7Blue-T vector by the TA cloning method to determine its structure.

It became evident that human OCTN2 contains an open reading frame (ORF) encoding a protein consisting of 557 amino acid residues. Fig. 3 compares amino acid sequences of human OCTN1 and human OCTN2. Both showed overall amino acid homology as high as about 76%. In addition, one consensus sequence (160 to 176) of sugar transporter was present in the amino acid sequence of human OCTN2 like human OCTN1. These facts indicated that human OCTN2 can be a novel transporter that is structurally related to human OCTN1. Furthermore, a consensus sequence (218 to 225) of the ATP/GTP binding site was also present in the amino acid sequence of human OCTN2 like in human OCTN1.

Example 5 Northern analysis

Northern analysis was performed using about 900 bp human OCTN2 cDNA as a probe which was obtained by PCR with a set

of 631R S4 primer (5'-GTGCTGTTGGGCTCCTTCATTTCA-3', SEQ ID NO: 13) and 631R A1 primer (5'-AGCTGCATGAAGAGAAGGACACTG-3', SEQ ID NO: 14) in the same manner as for human OCTN1. The results are shown in Fig. 4. Although the expression pattern of human OCTN2 partly overlapped with that of human OCTN1, human OCTN2 differs from human OCTN1 in that the former was very intensely expressed in the kidney among fetal tissues, while the latter was strongly expressed also in cancer cell strains such as K-562, HeLa S3, SW480, etc. as well as the kidney, indicating that OCTN1 and OCTN2 may be involved in transport of substances such as carcinostatics in these cancer cells.

Example 6 Forced expression of human OCTN1 in human fetal kidney cells (HEK293) and its activity determination

Phage DNAs were extracted from positive phage clones obtained by screening the clones by the plaque hybridization method using the QIAGEN Lambda Kit (QIAGEN). After the DNA insert was subcloned into the pUC18 vector, cDNA containing the entire ORF which was cleaved out with SmaI and EcoRI was integrated between the EcoRI site and the blunted HindIII site of an expression vector for mammalian cells, pcDNA3 (Invitrogen), to obtain an expression plasmid DNA, pcDNA3/OCTN1. Plasmid DNA was prepared by alkaline-SDS method using the QIAGEN PLASMID MAXI Kit (QIAGEN).

The human fetal kidney-derived cell strain, HEK 293 cells were transfected with the plasmid pcDNA3/OCTN1 and pcDNA3 vector containing no insert as a control by the calcium phosphate method. First, the plasmid DNA (10 μg), a

Hepes buffer solution (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM Dextrose, and 21 mM Hepes pH 7.1) (1 ml), and 2 M $CaCl_2$ (62.5 μl) were combined and allowed to stand at room temperature for 30 min or more to form calcium phosphate coprecipitates. After cells were plated on 10-cm plates at 1.5 x 10^6 cells per plate and cultured for 24 h, the calcium phosphate coprecipitates were added thereto, and the cells were further cultured for 24 h. Then, plates were washed with phosphate buffered saline (PBS), and the cells were further cultured for 24 h after the addition of fresh culture medium.

Transport experiment was performed using cells transfected with the plasmid DNA or untreated cells according to the following procedures. Cells were detached from plates using a rubber policeman, suspended in a transport buffer (containing 125 mM NaCl, 4.8 mM KCl, 5.6 mM (+)-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, and 25 mM Hepes pH 7.4), and pre-incubated for 20 min. An appropriate amount of each labeled substrate ([^{14}C]TEA (tetraethylammonium) (NEN), [^{3}H]carnitine (L-carnitine hydrochloride) (Amersham), [^{3}H]PCG (benzylpenicillin) (Amersham), [^{3}H]quinidine (ARC), or [^{3}H]pyrilamine (mepyramine) (Amersham)) was then added to the cell suspension, and the resulting mixture was incubated at 37°C for a predetermined period of time. Incubated cells were overlaid on a silicon layer prepared by layering a mixture of silicon oil and liquid paraffin (specific gravity = 1.022) on a 3 M KCl layer, and separated by centrifugation. Radioactivity of cells was measured to determine the into-the-cell transport activity. In this case, 1 x 10^6 cells were used as one point of cells. HEK 293 cells were

cultured in Dulbecco's MEM containing 10% fetal calf serum (FCS) in an atmosphere of 5% carbon dioxide at 37°C.

First, the transporter capacity was measured in the cells transfected with pcDNA3/OCTN1 and untreated cells using TEA as a substrate (Fig. 5). A reaction time-dependent TEA uptake into the human OCTN1-transfectd cells was clearly observed. This uptake was not observed in untreated cells. Next, effects of the addition of unlabeled TEA on the labeled substrate uptake in this system (cold inhibition) was examined (Fig. 6). A decrease in the apparent uptake of the labeled substrate was clearly seen depending on the concentration of cold TEA added. In this experiment, almost no uptake of the substrate into cells was observed in cells transfected with the pcDNA3 vector containing no insert (Mock) used as a control like in untreated cells used, clearly indicating that this uptake phenomenon is due to the transfection of the cells with human OCTN1. Next, to obtain the Km (Michaelis constant) value of human OCTN1 to TEA, the uptake of ^{14}C-TEA with various concentrations was measured (Fig. 7). From Lineweaver-Burk reciprocal plot of the net uptake obtained by subtracting the amount of the uptake in Mock cells from that in the human OCTN1-transfected cells, the Km value of 0.44±0.04 mM was obtained with the maximal velocity, Vmax of 6.68±0.34 (nmol/3 min/mg). Next, the transport capacity of human OCTN1 for other substrate than TEA was examined (Fig. 8). When the transport capacity was measured using labeled organic cations such as labeled carnitine, quinidine, and pyrilamine, a significant increase in the uptake of these compounds was clearly observed in human OCTN1-transfected cells as compared with Mock cells,

clearly indicating that these organic cations can serve as substrates for human OCTN1. However, no significant increase in the uptake of an organic anion, PCG (benzylpenicillin), was observed.

Example 7 Activity measurement of human OCTN1 using *Xenopus* oocytes

cRNA was synthesized *in vitro* using T7 RNA polymerase with pcDNA3/OCTN1 as a template. This cRNA was diluted to the concentration of 0.3 ng/nl, and its 50-nl (15 ng) aliquot was injected into a single oocyte. As a control, 50 nl of distilled water was injected. These oocytes were cultured for 3 days, and then used for the transport experiment. After being preincubated in an uptake buffer (0.05% Tween 80, 100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM Hepes pH 7.4) at 25°C for 20 min, the oocytes were transferred to the uptake buffer containing 0.5 ml of labeled substrate to initiate the uptake. After the incubation at 25°C for 1 h, the oocytes were washed in the ice-cold uptake buffer three times to terminate the reaction. The oocytes were solubilized in 5% SDS and mixed with Cleasol I (a cocktail for liquid scintillation counter) (3 ml) to determine the radioactivity. The radioactivity of the uptake buffer which contained the labeled compound at the time of incubation (external solution) (10 μl) was also similarly measured. The ratio of the radioactivity (dpm value) in the oocytes to that (dpm value) in the external solution was used as the uptake activity.

Human OCTN1 also expresses the transport capacity for organic cations such as quinidine, mepyramine and carnitine, as well as TEA in this transport experiment system using *Xenopus* oocytes (Fig. 9).

Next, the transport capacity of human OCTN1 for carcinostatics, etc. was examined. The results revealed that human OCTN1 has the activity to transport actinomycin D, etoposide, vinblastine, and daunomycin (Fig. 10). These results strongly indicate that OCTN1 would be involved in the into-the-cell translocation mechanism (mechanism for absorption by cells) for these drugs, which have been clinically used as carcinostatics. By designing and screening drugs utilizing the substrate specificity of OCTN1 so as to be readily recognized by this transporter, it would be possible to efficiently develop useful drugs that can be readily absorbed by the cells.

Example 8 Forced expression of human OCTN2 in HEK cells and its activity measurement

The expression plasmid DNA for human OCTN2 in mammalian cells was prepared as follows.

A single-stranded cDNA was synthesized from poly(A)$^+$ RNA derived from the human fetal kidney (CLONTECH) using the SuperScript™ II reverse transcriptase (GIBCO BRL). PCR was performed using the thus-obtained cDNA as a template under the following conditions to amplify 5'- and 3'-end fragments of human OCTN2.

For the amplification of 5'-end fragment (about 800 bp) of human OCTN2, OCTN2 3 primer (5'-GATGGATCCCGGACGGTCTTGGGTCGCCTGCTG-3', SEQ ID NO: 16) and

OCN2 4 primer (5'-GATGGATCCAAATGCTGCCACATAGTTGGAGAT-3', SEQ ID NO: 17) were used. PCR was carried out using DNA polymerase ExTaq (TaKaRa) and dNTPs (150 μM 7-deaza dGTP, 50 μM dGTP, 200 μM dATP, 200 μM dTTP, and 200 μM dCTP) according to the following conditions:94°C for 2 min; 35 cycles of 94°C for 30 s, 63°C for 1 min, and 72°C for 2 min,; and 72°C for 10 min. For the amplification of 3'-end fragment (about 1.2 kbp) of human OCTN2, OCTN2 7 primer (5'-GATGGATCCATGGGCATGCAGACAGGCTTCAGC-3', SEQ ID NO: 18) and OCTN2 8 primer (5'-GATGGATCCTTCCTCTTCAGTTTCTCCCTTACT-3', SEQ ID NO: 19) were used. PCR was carried out using DNA polymerase ExTaq (TaKaRa) and dNTPs (200 μM dGTP, 200 μM dATP, 200 μM dTTP, and 200 μM dCTP) according to the following conditions:94°C for 2 min; 35 cycles of 94°C for 30 s, 63°C for 30 s, and 72°C for 2 min,; and 72°C for 10 min.

These fragments were respectively electrophoresed on agarose gel, excised from the gel, purified, and subcloned into the pT7Blue-T vector. Clones having no PCR error were selected by sequencing, and clones from both fragments were ligated at the PstI site in the overlapping region. Each ligated fragment was eventually incorporated into the BamHI site of the pcDNA3 vector, and used as the expression plasmid DNA pcDNA3/OCTN2.

HEK cells were transfected with pcDNA3/OCTN2, the pcDNA3 vector containing no insert (Mock), or pcDNA3/OCTN1 by the method described in Example 6 to perform transport experiments. It was proved that human OCTN2 has a high capacity to efficiently transport carnitine (Fig. 11). On the other hand, human OCTN2 hardly transported TEA, which

were efficiently transported by human OCTN1, revealing that they clearly differ in their substrate specificities.

Next, Na^+ dependence of human OCTN2-mediated carnitine transport was examined using a transport buffer in which Na^+ was replaced with K^+ (Fig. 12). The result showed that carnitine transport mediated by human OCTN2 completely depended on the presence of Na^+, indicating that OCTN2 is a symport type transporter that transports substrates and Na^+ in the same direction.

Example 9 Cloning of mouse OCTN1

Data base search using human OCTN1 cDNA sequence detected several Expressed Sequence Tags (ESTs) derived from mouse, which had very high homology to the human OCTN1 cDNA sequence. Based on these EST sequences, MONL 1 primer (5'-CGCGCCGAATCGCTGAATCCTTTC-3', SEQ ID NO: 20) and MONA 4 primer (5'-AGGCTTTTGATTTGTTCTGTTGAG-3', SEQ ID NO: 21) were prepared. PCR was performed using a set of these primers and cDNA prepared from poly(A)$^+$ RNA derived from the mouse kidney as a template. As a result, fragments of about 2 kbp were amplified. These fragments were electrophoresed on agarose gels, excised from the gels, purified, and subcloned into the pT7Blue T vector (Novagen) by the TA cloning method. The sequence of mouse OCTN1 was determined by sequencing plural clones. The nucleotide sequence of cDNA thus determined is shown in SEQ ID NO: 23, and amino acid sequence of the protein encoded by the cDNA in SEQ ID NO: 22.

Example 10 Cloning of mouse OCTN2

First, MONB 20 primer (5'-CCCATGCCAACAAGGACAAAAAGC-3', SEQ ID NO: 24) was prepared from the sequence of human OCTN2 cDNA. The Marathon-Ready™ cDNA derived from the mouse kidney (CLONTECH) was used as a template for the 5'-Rapid Amplification of cDNA ends (RACE) to clone the 5'-end sequence upstream of the primer. Next, data base search was performed using human OCTN2 nucleotide sequence to detect several ESTs derived from mouse, which had a very high homology with human OCTN2. MONB 26 primer (5'-ACAGAACAGAAAAGCCCTCAGTCA-3', SEQ ID NO: 25) was prepared from these EST sequences. MONB 6 primer (5'-TGTTTTTCGTGGGTGTGCTGATGG-3', SEQ ID NO: 26) was prepared from the sequence obtained by the 5'-RACE. PCR was performed using this primer and MONB 26 primer and cDNA prepared from poly(A)$^+$ RNA derived from the mouse kidney as a template to amplify the 3'-end fragments. The sequence of mouse OCTN2 was determined by sequencing directly of after subcloning respective fragments. The nucleotide sequence of the cDNA thus determined is shown in SEQ ID NO: 28, and amino acid sequence of the protein encoded by the cDNA in SEQ ID NO: 27.

Example 11 Tissue expression analysis of mouse OCTN1 and mouse OCTN2

The expression amount of mouse OCTN1 and mouse OCTN2 genes in various tissues was examined by RT-PCR using a mouse Multiple Tissue cDNA (MTC) panel (CLONTECH) (Fig. 13). Primers used are MONL 1 and MONA 4 for mouse OCTN1, and MONB 6 and MONB 26 for mouse OCTN2. As a

result, the high level expression of mouse OCTN1 was detected in the kidney and liver, while that of mouse OCTN2 in the kidney, liver, and 7-days old embryo.

Industrial Applicability

This invention provides a family of novel organic cation transporter genes and proteins encoded by these genes. Transporter proteins of this invention are useful for developing newly designed drugs that can be transported mediated by these proteins, and pharmaceuticals for disorders caused by functional abnormalities of the proteins.

What is claimed is: